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PROCESS FOR PRODUCING CATIONIC PEPTIDES FROM BIOLOGICAL FLUIDS

Field of the Invention

The present invention relates to a process for the production of cationic peptides of interest from proteins which occur in biological fluids. This process involves the separation of charged proteins from fluids such as milk, whey or blood, by ion exchange chromatography, followed by further steps comprising fragmenting th s proteins and selecting the cationic peptides of interest.

Background of the Invention

In recent years, it has become widely recognised that many organisms, from prokaryotes to humans, use peptides as part of their host defense system. Among these, cationic peptides form an important class showing a variety of antimicrobial properties. Different peptides may have antibacterial, antiendotoxic, antibiotic-potentiating or antifungal properties, and so they are being developed for use as a novel class of antimicrobial agents and as the basis for making transgenic disease-resistant plants and animals. For a review, see, e.g., R.E.W. Hancock and R. Lehrer, *Tibtech February 1998*, 16:82-88.

W. Lee Maloy and U. Prasad Kari, *Biopolymers (Peptide Science)* 37:105-122 (1995) report that the structures of these peptides from insects, horseshoe crabs, frogs, and mammals are known to have in common features of a net cationic charg due to the presence of multiple Arg and Lys residues and in most cases the ability to form amphipathic structures. Despite very diverse peptide sequences and structure , most host defense peptides appear to act by a direct lysis of the pathogenic cell membrane. Their basic structure facilitates their interaction with the cell membrane, and their amphipathic character allows them to be incorporated into the membrane, ultimately disrupting its structure. These authors divide the host defense peptides in two structural classes, linear peptides and cyclic peptides with one to three disulfid bonds. A further tentative classification of antimicrobial peptides into four distinct groups is given by D. Destoumieux *et al., J. Biol. Chem.* (1997) 45:28398-28406. These groups are based on amino acid sequences, secondary structures, and

functional similarities: (i) linear basic peptides forming amphipathic α-h lices, (ii) peptides with one to six intramolecular disulfide bridges, (iii) proline-rich peptides, and (iv) glycine-rich antimicrobial peptides or polypeptides (9-30 kDa). It is referred to that the mode of action, the broad activity, the molecular diversity, and the noncytotoxicity of all these circulating antimicrobial peptides make them very attractive as therapeutic agents for pharmaceutical or agricultural applications.

W.E. Robinson *et al, J. Leukoc. Biol.* (1998) 63:94-100, report anti-HIV-1 activity of indolicidin, an antimicrobial tridecapeptide amide isolated from the cytoplasmic granules of bovine neutrophils. Similarly, D. Winder *et al., Biochem. Biophys. Res. Comm.* (1998) 242:608-612, disclose that the expression of genes encoding the (cationic) antimicrobial peptides cecropin and mellitin has an antitumour effect in human cells..

K. Natarajan and J.A. Cowan, *Chemistry and Biology* (1998) 5:147-154 postulate on the basis of the solution structure of a synthetic lytic peptide corresponding to the amino terminus of human perforin, that the strong electrostatic interaction between the cationic region of said peptide and the lipid headgroups probably weakens the membrane, facilitating insertion of the relatively neutral/ hydrophobic stretch of said peptide, and is representative of the lytic pathway. It is stated that this structural model is probably relevant to understanding the mechanisms of other cationic antimicrobial peptides.

T. Wieprecht *et al., Biochemistry* (1997) 36:12869-12880 report on the influence of the angle subtended by the positively charged helix face on the membrane activity of amphipathic, antibacterial peptides. An amphipathic, mostly α-helical conformation and a positive net charge have been recognised as major structural motifs which determine the membrane disturbing activity. Enhancement of the total cationic peptide charge often results in a higher antibacterial activity.

Lactoferrin is an iron-binding protein that is present in the whey fraction of milk. This protein is a main antimicrobial component of milk and is thought to contribute to the protection of infants from infectious diseases. A large number of studies has demonstrated its strong bacteriostatic effect and sometimes its bact ricidal effect and its antiviral activity. Lactoferrin may mediate som of the ffects of inflammation and has a role in regulating various components of the immune

system. For a review, se , e.g., L. Sanchez *et al., Arch. Dis. Child.* 67:657-661.

Some bioactive peptides from lactoferrin have been reported. For example, in the early 90s a tetrapeptide of human lactoferrin was described as an inhibitor of platelet aggregation, of fibrinogen binding to ADP-activated platelets and of serotonin release: it was antithrombotic in rats, guinea-pigs and in dogs. See E. Mazoyer *et al., Eur. J. Biochem.* (1990) 194:43-49; L. Drouet *et al., Nouv. Rev. Fr. Hematol.* (1990) 32:59-62; G. Wu *et al., Haemostasis* (1992) 22:1-6.

Some peptides of human or bovine lactoferrin possess antibacterial properties. It was found that a pepsin-generated hydrolysate of lactoferrin displayed bactericidal properties that were more potent than those of undigested lactoferrin. Also, a potent antibacterial peptide fragment from the N-terminal part of lactoferrin, named lactoferricin, was isolated from that hydrolysate. See, e.g., W. Bellamy *et al., J. Appl. Bacteriol.* (1992) 73:472-479 and *Biochim. Biophys. Acta* (1992) 1121:130-136; M. Tomita *et al., J. Dairy Sci* (1991) 74:4137-4142; K. Yamauchi *et al., Infect. and Immun.* (1993) 61:719-728; and E.M. Jones *et al., J. Appl. Bacteriol.* (1994) 77:208-214.

Y.-C. Yoo *et al., Biochem. Biophys. Res. Comm.* (1997) 297:624-628 report on the activity of bovine lactoferricin to induce apoptosis in THP-1 human monocytic leukemic cells.

Z.-Y. Qian *et al., Biochim. Biophys. Acta* (1995) 1243:25-32 disclose the isolation and characterisation pepsin hydrolysates of sheep and human lactoferrin and their inhibiting effect on platelet aggregation. It was *inter alia* found that a positive charge and a certain conformation of peptides are very important for the binding of lactoferrin to their receptors at the surface of platelets.

The prior art referred to above and the references cited therein, th disclosure of which is herein incorporated by reference, show abundantly the interest in the biological properties including the structure-activity relationship of naturally occurring peptides or peptide fragments, in particular in relation to the immune responses of humans and animals.

A variety of methods for the preparation and isolation of lactoferrin and its hydrolysates hav b en described in th prior art. See, for xample, WO 89/04608, WO 93/02098, WO 93/13676, EP-A-0253395, EP-A-0556083, FR 2613725 and NL

8601814, wher in *inter alia* techniqu s are applied for the s paration of charged molecules which include cation exchange means.

EP-A-0519726 discloses a process for large-scale production of an antimicrobial peptide in high purity wich comprises the steps of first isolating the protein containing the peptide of interest, contacting a mixture of peptides containing the antimicrobial peptide obtainable by hydrolysing bovine lactoferrin or bovine milk protein containing lactoferrin, with a hydrophobic chromatographic medium or cation-exchange chromatographic medium to adsorb the antimicrobial peptide, washing the medium to elute peptides other than the antimicrobial peptide, desorbing the antimicrobial peptide in solution from the washed medium, and, optionally, desalting the desorbed antimicrobial peptide solution, whereby the antimicrobial peptide of at least 90% by weight purity is obtained. This process is rather complex and therefore expensive, and one can really wonder if for many applications an antibacterial product based on lactoferrin having a somewhat lower specific activity as compared to lactoferrin but produced in a relatively simple and therefore cheaper way, would not suffice.

In view of the increased interest in cationic peptides and their useful and promising properties, there is therefore an increasing need for an efficient way to produce such peptides. The present invention aims to provide such a method which is both relatively simple and cheap.

Brief Description of the Drawings

Figure 1 is a schematic and illustrative representation of a preferred embodiment of the process according to the present invention.

Figure 2 is a RP-HPLC chromatogram of the active product obtained after hydrolysis of bovine α_{S2}-casein bound to the cation-exchange membrane (starting from a solution of α_{S2}-casein).

Figure 3 is a RP-HPLC chromatogram of the active product obtained after hydrolysis of goat whey protein bound to the cation-exchange membrane (starting from microfiltered goat whey).

Figure 4 is RP-HPLC chromatogram of the active product obtained after hydrolysis of sheep whey protein bound to the cation-exchange membrane (starting

from microfiltered sheep whey).

Figure 5 is a RP-HPLC chromatogram of the active product obtained after hydrolysis of bovine whey protein bound to the cation-exchange membrane (starting from microfiltered bovine whey).

Figure 6 is a RP-HPLC chromatogram of the active product obtained after hydrolysis of bovine lactoferrin bound to the cation-exchange membrane (starting from a solution of bovine lactoferrin).

Figure 7 is a RP-HPLC chromatogram of (a) the cell-free supernatant containing nisin A precursor, (b) the active product obtained after hydrolysis of nisin A precursor bound to the cation-exchange membrane.

In all Figures the observed masses of identified peptides are indicated.

Summary of the Invention

In accordance with an aspect of the present invention, there is provided a process for the production of at least one cationic peptide of interest from a biological fluid which comprises the steps of:

a) contacting said biological fluid comprising one or more proteins which contain said cationic peptide of interest with an ion exchange chromatographic medium to adsorb said protein containing said cationic peptide,

b) subjecting said adsorbed material to hydrolysis to fragment said protein and produce said cationic peptide which remains substantially adsorbed,

c) washing the medium to remove unbound material,

d) desorbing said cationic peptide from said chromatographic medium, and, optionally,

e) further purifying said desorbed cationic peptide of interest.

Preferably, the medium is also washed before the hydrolysis step to remov unbound material. The ion exchange chromatographic medium is preferably a cation exchanger and most preferably a membrane cation exchanger. The hydrolysis is preferably carried out enzymatically. Suitable enzymes include, e.g., pepsin, chymosin, chymotrypsin and thermolysin. Preferred biological fluids include milk, whey

and other milk products, blood, blood serum, culture cells, extracts from cultur cells, and plant cells.

In another aspect of the invention various cationic peptides are provided which are obtainable by the present process.

In still another aspect of the invention the cationic peptides are suitable for use as pharmaceutical compositions for various therapeutic and diagnostic purposes, for example based on antimicrobial, antiviral or antitumour activity.

These and other embodiments and advantages of the invention will become apparent to those skilled in the art upon review of the following detailed description and claims.

Detailed Description of the Invention

Asymmetric clustering of basic residues has been observed in various peptides that show an affinity for biological membranes. These cationic peptides have been shown, besides other activities, to kill sensitive microorganisms by inducing an increase in cell membrane permeability. The isolation of these peptides from prot in hydrolysates is generally laborious, because it implies first the isolation of th precursor protein and then the purification of the cationic peptides from the enzymatic protein hydrolysate.

As used herein, the term "peptide" generally refers to a protein fragment without being bound to a certain size, thus including "oligopeptide" and "polypeptide". The term "peptide of interest" as used herein refers to a peptide having any kind of activity, and most preferably a biological activity.

In accordance with the present invention it has surprisingly been found, after extensive research and experimentation, that a cationic peptide of interest or a fraction enriched in such cationic peptides of interest can be directly and efficiently obtained from a biological fluid in which proteins are contained which comprise such peptide(s) of interest, by a process comprising the steps of (i) contacting said biological fluid with an ion-exchanger, preferably a cation-exchanger, (ii) hydrolysing *in situ* the selectively bound protein, i.e. hydrolysis on the ion-exchanger, without the need for intermediate isolation of the pr cursor protein(s) of interest, (iii) washing out the unbound peptid s, (iv) s lectiv ly eluting the cationic peptide or peptides from the

ion-exchanger, and, optionally (v) further purifying the resulting cationic peptide or peptides.

The starting material used in the present process is any protein or mixture of proteins containing cationic domains within their sequences. The process is particularly suitable for biological fluids such as milk, whey or other milk fractions, blood or blood serum, cell cultures or cell culture extracts, or solutions of plant proteins. However, it will be understood that any fluid comprising proteins which contain a cationic peptide of interest may be used. For the purpose of the pres nt invention, such fluids are also comprised within the term "biological fluid".

The starting material is dissolved or dispersed in water or buffer solution, preferably low ionic strength buffer at the appropriate pH to bind the protein of interest to the ion-exchanger. Usually, no purification step is required or needed prior to contacting the solution or dispersion with the ion-exchanger. Any insoluble material, if present, can be removed in a usual manner, such as by microfiltration, ultrafiltration or centrifugation.

Ion-exchange procedures have been decribed for the isolation of protein/ peptide components from fluids. For instance, lactoferrin and lactoperoxidase from cheese whey using bead-based ion-exchange. See the references referred to above.

Either a strong or a weak cation or anion exchange medium (resin, gel or membrane) can be used for the concentration of the protein of interest employing continuous-flow or batch-wise chromatographic techniques. A variety of groups can be chosen for use in cation exchangers, such as carboxymethyl, methyl sulphonate, sulphopropyl or sulfonic acid. The matrix is usually based on inorganic compounds, synthetic resins, polysaccharides, etc., a strongly acidic cation-exchange membrane being preferred. In such a membrane, functional groups are covalently attached to th inner surface of large pore size, non-compressible and chemically very stable microporous cellulose membrane (e.g. Sartorius).

The bound protein is then hydrolysed, preferably by enzymatic hydrolysis. However, acid or alkaline hydrolysis, optionally in combination with enzymatic hydrolysis can also be employed, if desired. The hydrolysis is carried out *in situ*, i.e. without isolating the selectiv ly bound protein. The medium is preferably washed with

water and/or an appropriate buffer solution prior to hydrolysis in order to substantially remove unbound materials.

In a preferred embodiment of the invention the selectively bound protein is enzymatically digested with pepsin. However, any protease (each at or near its optimum pH) which is capable of cleaving the protein of interest bound to the ion exchanger to generate peptides of interest may be used. Suitable enzymes include, for example, chymosin (pH approx. 3.0), chymotrypsin and thermolysin (both at neutral pH).

Washing out the ion-exchanger and preferably the membrane to remove other protein fragments, i.e. mainly non-cationic peptides, is usually commenced immediately after hydrolysis of the protein of interest. This step can be suitably perfomed with water and/or a buffer solution, preferably low ionic strength buffer, at low or neutral pH, which pH is preferably about one pH unit lower than the pI of the bound cationic peptide or peptides. For instance, the washing out of these fragments can be performed with water or 10 mM sodium phosphate buffer, or alternatively a volatile buffer, e.g. 10 mM ammonium bicarbonate buffer. If desired, this by-product fraction is dried by conventional means, e.g. freeze-drying or spray-drying, and may be further employed.

The cationic peptides bound to the membrane are then eluted in a usual way employing one or more appropriate buffer solutions, preferably volatile buffers, e.g. ammonium solutions of different concentrations thus avoiding a desalting step. If necessary, high ionic strength buffers, e.g. 2 M sodium chloride in phosphate buffer can be employed.

The elution step is suitably followed by further purification of the resulting cationic peptide fraction in a manner known per se. For example, if necessary, desalting of the solution containing the cationic peptide or peptides is suitably carried out by techniques such as ultrafiltration, dialysis, electrodialysis with membranes of suitable cut-off, gel permeation chromatography, hydrophobic interaction chromatography, or high performance liquid chromatography. If desired, this latter technique can be employed for further purification of the cationic peptide or peptides.

In a typical example, the process according to the present invention was successfully appli d to the small scal production of antibacterial peptid s derived

from lactoferrin (LF), using chees whey as starting material. Cheese whey from different species (cow, goat or sheep), or a mixture thereof, was employed and this resulted in a fraction of peptides with much higher activity (at least 12-fold more potent) than the undigested LFs. However, it will be clear to those skilled in the art that the present method can be successfully used with any proteins containing cationic fragments, which proteins may or may not be present in complex mixtures in biological fluids.

The chromatography step in the method according to the present invention is preferably carried out using ion-exchange membranes, but also other ion-exchange chromatography techniques can be employed, either in one or more columns or batchwise. Membrane-based processes are generally preferred since they are more rapid by allowing a higher flow rate (approx. 20-fold) as compared with bead-based systems. In addition, these membrane-based processes can be easily up-scaled to gram or even kilogram quantities.

The process according to the present invention is relatively simple and therefore generally economically and technically more attractive than the prior art methods. It provides in high yields cationic peptides with a selected activity of interest without the need of intermediate purification of the precursor protein. In a preferred embodiment of the invention, the process is used for the production of lactoferricin and derivatives thereof from milk or whey from an appropriate source (human or animal, such as bovine, goat, sheep, horse), having desired properties. Also casein, e.g. purified bovine α_{s2}-casein is a suitable and preferred source for the production of cationic peptides.

In a further aspect of the present invention, there are provided novel cationic peptides, which are obtainable by the invented process. Biologically active peptides are the preferred choice. Biological activities of interest which make these peptides particularly useful include antimicrobial, antiviral, antitumour, anti-inflammatory and antithrombotic activity. Evidently, the biological activity *inter alia* depends on the nature of the starting biological fluid and the proteins contained therein. Alternatively, these novel peptides can be produced by other methods known in the art, such as by ch mical synthesis or microbiologically or by plants.

Therefore, the invention furth r provides a cationic peptide of interest, obtainable by the method of the present invention, as described before, having an amino acid sequence selected from the following sequences (1) - (7), or derivativ s thereof having an amide at the carboxy end thereof, which derivatives do not interfere with or abolish any biological properties of the peptide:

(1) VYQHQKAMKPWIQPKT (= SEQ ID NO.: 1)
(2) VYQHQKAMKPWIQPKTKVIPY (= SEQ ID NO.: 2)
(3) VYQHQKAMKPWIQPKTKVIPYVRY (= SEQ ID NO.: 3)
(4) VYQHQKAMKPWIQPKTKVIPYVRYL (= SEQ ID NO.: 4)
(5) PEWSKCYQWQRRMRKLGAPSITCIRRTSA (= SEQ ID NO.: 5)
(6) TQRKTRNGFRVPLARE (= SEQ ID NO.: 6)
(7) APRKNVRW (= SEQ ID NO.: 7)

Although the process according to the present invention has been typically disclosed for the production of cationic peptides, since the process involves cation exchange chromatography followed by instant hydrolysis, it will be clear to those skilled in the art that this process is not restricted to cationic peptides. Using the same principle, but other types of ion exchangers, for example anion exchangers, followed by appropriate hydrolysis, the method is also suitable for the production of peptides which are differently charged.

The invention will be further illustrated by the following methods and examples which are not to be construed as limiting the invention in any respect.

Materials and methods

Analytical Procedures

1. Reversed-phase high performance liquid chromatography (RP-HPLC)

Analytical RP-HPLC was carried out using two M 6000A pumps in combination with a high-sensitivity accessory block (Waters Ass.), an ISS-100 injector (Perkin-Elm r), a Waters Model 680 gradient controller and a Kratos 783 detector (Kratos Analytical). A 250 mm x 4.6 mm Widepore C_{18} column (Bio-Rad Laboratori s)

was used with a C_{18} cartridge (Bio-Rad) as guard column. Th equipment was linked to a data acquisition and processing system (Turbochrom, Perkin-Elmer). Solvent A was a mixture of acetonitrile-water-trifluoroacetic acid (TFA) (100:900:1, v/v/v) and solvent B contained the same components (900:100:0.8, v/v/v).

Peptides were eluted using a linear gradient of solvent B in A from 0% to 50% over 70 min at a flow rate of 0.8 ml/min. The absorbance of the eluent was monitored at 220 nm. Sample concentrations were approx. 1 mg/ml and injection volumes 50 µl.

2. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS)

The mass of each purified peptide was assessed by mass spectrometry using a Quattro II triple quadrupole instrument (Micromass). Samples were dissolved in 50% acetonitrile, 0.3% formic acid and analysed by infusion with a syringe pump type 22 (Harvard Apparatus, South Natick, MA, USA) of the sample solution in 50% acetonitrile at 4 µl/min through the electrospray interface. Nitrogen was used as a nebulizing and drying gas. The capillary was held at 3.9 kV; the cone voltage was 30 V. MS/MS was achieved using $5x10^{-3}$ mbar of argon in the second quadrupol . Automated peak recognition and daughter ion scanning were performed using the Masslynx software version 2.2 (Micromass) on a Windows NT workstation.

3. N-terminal sequence analysis

The N-terminal part of the purified peptides was identified by sequence analysis with a gasphase sequenator (Model 470A, Applied Biosystems Inc.) using 25% TFA in water as conversion agent. The resulting phenythiohydantoin (PTH) amino acids were analysed on-line by RP-HPLC with a PTH analyser (Model 120A, Applied Biosystems Inc.) using a PTH C-18 column (2.1 mm x 220 mm) (Applied Biosystems Inc.).

4. Assay for antibacterial activity

Antibacterial activities of the peptide mixtures and/or isolated peptides wer determined by a plat diffusion assay using *Micrococcus flavus* DSM 1790 as indicator organism. *M. flavus* was grown in MF broth (0.1% sucrose, 1% peptone,

0.3% meat extract, 0.2% NaCl, 0.15% yeast extract pH 7.0). Peptide mixtures were tested at concentrations from 0.2 mg/ml to 50 mg/ml in bidistilled water.

A microtiter method was used to determine the minimal inhibitory concentrations (MICs) of the peptides in liquid media against various microorganisms, viz. *E. coli* (2 different strains), *Listeria innocua, Bacillus cereus, Micrococcus flavus, and Streptococcus thermophilus.*

Example 1

Preparation of a peptide mixture with antibacterial activity from α_{S2}-casein

Bovine α_{S2}-casein isolated from milk was dissolved in bidistilled water at a concentration of 0.1 mg/ml and the pH was adjusted to 3.0 by addition of 1 M HCl. Prior to use, the cation-exchange membrane (Sartorius Sartobind S MA 100; Sartorious GmbH, Germany) was pre-equilibrated with acidulate bidistilled water (pH 3.0). The process was monitored by a UV detector with 2 mm lightpath flow cuvett (model EM-1 Econo UV Monitor, Bio-Rad). 1000 ml of the α_{S2}-casein solution was pumped through the cation-exchange membrane at room temperature and at a flow rate of 20 ml/min. The cation-exchange membrane was then washed with acidified water (pH 3.0) to remove unbound material. The α_{S2}-casein bound to the membrane was hydrolysed for 6 hours at 37 °C by recycling at 20 ml/min, at reverse flow, 100 ml of an aqueous solution (25 mg/ml, pH 3.0) of porcine pepsin (Sigma Chemical Co.). The cation-exchange membrane was rinsed with acidulated water pH 3.0 followed by 10 mM ammonium bicarbonate buffer with formic acid (pH 7.0) until baseline level was achieved. Finally, the cationic antibacterial peptides were desorbed from the membrane with 7 M ammonia solution which was freeze-dried to obtain a powdered active product. This active product was monitored by reversed-phase high performance liquid chromatography (RP-HPLC) (Fig. 1), and the peptides were identified by mass spectrometry (MS) and tandem mass spectrometry (MS/MS). The masses and amino acid sequences of major peptides obtained in this product are summarised in Table 1. After extensive hydrolysis (overnight), the ratio among the four peptides was shifted towards the shortest sequence (mass 1982.9).

Tabl 1

Masses and amino acid sequences of the cationic antibacterial peptides obtained after hydrolysis of α_{s2}-casein on the cationic-exchange membrane

Observed mass	Theoretical mass	Sequence
1982.9	1983.4	VYQHQKAMKPWIQPKT
2583.8	2584.1	VYQHQKAMKPWIQPKTKVIPY
3002.2	3002.6	VYQHQKAMKPWIQPKTKVIPYVRY
3115.7	3115.8	VYQHQKAMKPWIQPKTKVIPYVRYL

Example 2

Preparation of a peptide with antibacterial activity from goat whey

Prior to its use, the cation-exchange membrane described in Example 1 was pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The process was monitored by a UV detector with 2 mm lightpath flow cuvette (model EM-1 Econo UV Monitor, Bio-Rad). 1000 ml of microfiltered goat cheese whey at pH 6.5 (hereaft r referred to as "starting material") was pumped through the cation-exchange membrane at room temperature and at a flow rate of 20 ml/min. The cation-exchange membrane was washed with acidified bidistilled water (pH 3.0) or alternatively, bidistilled water or 10 mM phosphate buffer to remove unbound material. The material bound to the cation-exchange membrane was hydrolysed overnight at 37 °C by recycling at 20 ml/min, at reverse flow, 100 ml of an aqueous solution (25 mg/ml, pH 3.0) of porcine pepsin (Sigma Chemical Co.). The cation-exchange membrane was rinsed with acidulated water pH 3.0 followed by a 7 M ammonia solution until baseline level was achieved. Finally, the strongly bound peptides were eluted with 2 M NaCl. The 2 M NaCl fraction was desalted using a Sep-Pak® C_{18} environmental cartridge (Wat rs Ass.) with a linear 0 to 100% gradient of acetonitrile in water. After acetonitrile evaporation, this fraction was freeze-dried to obtain a powdered active product. This active product was monitored by RP-HPLC (Fig. 2) and MS and the sequence of the major peptide in this fraction was derived from N-t rminal sequencing and MS data.

The mass and the sequence of the major peptide in this product are summaris d in Table 2.

Table 2

Mass and amino acid sequence of the cationic antibacterial peptide obtained after hydrolysis on the cationic-exchange membrane using goat cheese whey as starting material

Observed mass	Theoretical mass	Sequence
3493.3	3493.9	PEWSKCYQWQRRMRKLGAPSITCIRRTSA

Example 3

Preparation of a peptide with antibacterial activity from sheep whey

Experimental conditions were as described above for Example 2 with the exception that microfiltered sheep cheese whey (pH 6.5) was used as starting material. The active product obtained was monitored by RP-HPLC (Fig. 3) and MS and the sequence of the major peptide in this fraction was derived from N-terminal sequencing and MS data. The mass and the sequence of the major peptide in this product are summarised in Table 3. The data indicate that the active product was sheep lactoferrine peroxidase.

Table 3

Mass and amino acid sequence of the cationic antibacterial peptide obtained after hydrolysis on the cationic-exchange membrane using sheep cheese whey as starting material

Observed mass	Theoretical mass	Sequence
1929.0	1929.2	TQRKTRNGFRVPLARE

Example 4

Preparation of a peptide mixture with antibacterial activity from bovine whey

Experimental conditions were as described above for Example 2 with the exception that microfiltered bovine Gouda cheese whey (pH 6.5) was used as starting material. The active product obtained was monitored by RP-HPLC (Fig. 4) and MS and the sequence of the major peptides in this fraction was derived from N-terminal sequencing and MS data. The masses and the sequences of the major peptides in this product are summarised in Table 4.

Table 4

Masses and amino acid sequences of the cationic antibacterial peptides obtained after hydrolysis on the cationic-exchange membrane using bovine cheese whey as starting material

Observed mass	Theoretical mass	Sequence
1026.0	1026.2	APRKNVRW
3123.2	3123.8	FKCRRWQWRMKKLGAPSITCVRRAF
3194.3	3194.9	FKCRRWQWRMKKLGAPSITCVRRAFA

Example 5

Preparation of a peptide mixture with antibacterial activity from purified bovine lactoferrin

Experimental conditions were as described above for Example 2 with the exception that a solution of purified bovine lactoferrin in 10 mM sodium phosphate buffer (pH 7.0) was used as starting material. The active product obtained was monitored by RP-HPLC (Fig. 5) and MS and the sequence of the major peptides in this fraction was derived from N-terminal sequencing and MS data. The masses and the sequences of the major peptides in this product are summarised in Table 5.

Table 5

Masses and amino acid sequences of the cationic antibacterial peptides obtained after hydrolysis on the cationic-exchange membrane using a solution of purified lactoferrin as starting material.

Observed mass	Theoretical mass	Sequence
1026.0	1026.2	APRKNVRW
3123.2	3123.8	FKCRRWQWRMKKLGAPSITCVRRAF
3194.3	3194.9	FKCRRWQWRMKKLGAPSITCVRRAFA

Example 6

Preparation of the lantibiotic nisin A from a cell extract containing nisin A precursor

For the production of nisin A precursor, *Lactococcus lactis* strain MG1614 harbouring the plasmid pNZ9111 [Van der Meer *et al.*, J. Bacteriol. (1993) 175:2578-2588] was grown to the early stationary phase (final optical density at 600 nm 0.9-1.0, pH 6.2) in M17 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5 w/v % glucose and 10 mg/ml erythromycin. The culture was centrifuged for 20 min at 2,500 × *g* to remove lactococcal cells. The supernatant was again centrifuged for 30 min at 10,000 × *g* and, after 10-fold dilution with bidistilled water, the pH was adjusted to 4.5-4.8 with acetic acid.

Prior to use, the cation-exchange membrane (Sartorius Sartobind S MA 100; Sartorius GmbH, Göttingen, Germany) was equilibrated with 10 mM ammonium acetate buffer, pH 4.5. The process was monitored by a UV detector with a 2-mm lightpath flow cuvette (Model EM-1 Econo UV Monitor, Bio-Rad Laboratories, Richmond, CA, USA). 3 L of the 10-fold diluted cell-free supernantant was pumped through the cation-exchange membrane at room temperature and at a flow rate of 20 ml/min which was generated by a peristaltic pump (Verder-Vleuten b.v., Vleuten, The Netherlands). The cation-exchange membrane was then washed with 10 mM sodium phosphat buffer, pH 7.0, to remove unbound material. The nisin precursor bound to the membrane was hydrolysed for 15 min at 37 °C by r cycling, at 20 ml/min and at reverse flow, 50 ml of a solution of trypsin (Sigma Chemical Co., St. Louis, MO, USA)

(0.1 mg/ml) in 10 mM sodium phosphate buffer, pH 7.0. After hydrolysis, the membrane was rinsed with 10 mM sodium phosphate buffer, pH 7.0, until baseline level was achieved. Finally, nisin was desorbed from the cation-exchange membrane with 1 M KCl in phosphate buffer, pH 7.0. This fraction showed activity against *Micrococcus flavus* when tested by a plate diffusion assay. The active product was monitored by reversed-phase high-performance liquid chromatography (RP-HPLC) (Fig. 7), and the isolated nisin A component was identified by mass spectrometry.

The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(B) STREET: Kernhemseweg 2
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(F) POSTAL CODE (ZIP): 6718 ZB
(G) TELEPHONE: +31 318 659511
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(ii) TITLE OF INVENTION: Process for producing cationic peptides from biological fluids

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: bovine alpha-s2 casein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Val	Tyr	Gln	His	Gln	Lys	Ala	Met	Lys	Pro	Trp	Ile	Gln	Pro	Lys	Thr
1				5				10						15	

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: bovine alpha-s2 casein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Val Tyr Gln His Gln Lys Ala Met Lys Pro Trp Ile Gln Pro Lys Thr
1 5 10 15

Lys Val Ile Pro Tyr
20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: bovine alpha-s2 casein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Val Tyr Gln His Gln Lys Ala Met Lys Pro Trp Ile Gln Pro Lys Thr
1 5 10 15

Lys Val Ile Pro Tyr Val Arg Tyr
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: bovine alpha-s2 casein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Val Tyr Gln His Gln Lys Ala Met Lys Pro Trp Ile Gln Pro Lys Thr
1 5 10 15

Lys Val Ile Pro Tyr Val Arg Tyr Leu
20 25

(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: goat whey

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Pro Glu Trp Ser Lys Cys Tyr Gln Trp Gln Arg Arg Met Arg Lys Leu
1 5 10 15

Gly Ala Pro Ser Ile Thr Cys Ile Arg Arg Thr Ser Ala
20 25

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: sheep whey
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Thr Gln Arg Lys Thr Arg Asn Gly Phe Arg Val Pro Leu Ala Arg Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: bovine whey

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ala Pro Arg Lys Asn Val Arg Trp
1 5

Claims

1. A process for the production of at least one cationic peptide of interest from a biological fluid which comprises the steps of:

a) contacting said biological fluid comprising one or more proteins which contain said cationic peptide of interest with an ion exchange chromatographic medium to adsorb said protein containing said cationic peptide,

b) subjecting said adsorbed material to hydrolysis to fragment said protein and produce said cationic peptide which remains substantially adsorbed,

c) washing the medium to remove unbound material,

d) desorbing said cationic peptide from said chromatographic medium, and, optionally,

e) further purifying said desorbed cationic peptide of interest.

2. The process according to claim 1, further comprising, before step b), the step of washing the medium to remove unbound material.

3. The process according to claim 1 or claim 2, wherein the ion exchange chromatographic medium is a cation exchanger.

4. The process according to claim 3, wherein the cation exchanger is a membrane cation exchanger.

5. The process according to any one of claims 1 to 4, wherein the hydrolysis is carried out enzymatically.

6. The process according to claim 5, wherein one or more enzymes are used selected from the group of pepsin, chymosin, chymotrypsin and thermolysin.

7. The process according to any one of the preceding claims, wherein th biological fluid is s lected from the group of milk, whey, blood, blood serum, culture cells, extracts from culture cells, and plant cells.

8. A cationic peptide when prepared by a method according to any one of claims 1 to 7.

9. A cationic peptide, obtainable by the method of any one of claims 1 to 7, having an amino acid sequence selected from the following sequences (1) - (7), or derivatives thereof having an amide at the carboxy end thereof, which derivatives do not interfere with any biological properties of the peptide:

(1) VYQHQKAMKPWIQPKT

(2) VYQHQKAMKPWIQPKTKVIPY

(3) VYQHQKAMKPWIQPKTKVIPYVRY

(4) VYQHQKAMKPWIQPKTKVIPYVRYL

(5) PEWSKCYQWQRRMRKLGAPSITCIRRTSA

(6) TQRKTRNGFRVPLARE

(7) APRKNVRW.

10. Use of a cationic peptide as claimed in claim 8 or claim 9, for the preparation of pharmaceutical compositions, preferably with antimicrobial and/or antiviral and/or antitumour activity.

Abstract of the Invention

A process is provided for the production of at least one cationic peptide of interest from a biological fluid comprising one or more proteins which contain said cationic peptide, wherein said fluid is contacted with an ion exchanger to adsorb said protein including the cationic peptide domain, the adsorbed material is hydrolysed to produce said cationic peptide, the medium is washed to remove unbound material, the cationic peptide is desorbed from the ion exchanger and, optionally, further purified. Preferably, a membrane cationic exchanger is used and the hydrolysis is preferably carried out enzymatically. Also claimed are various cationic peptides, preferably with a biological activity, which are obtainable by the present process.

1/7
protein of interest
bioactive peptide
enzyme
Cation exchange membrane
1. Concentration of the protein of interest on the membrane
2. Enzymatic hydrolysis *in situ* of the protein and liberation of the bioactive peptide
3. Selective elution

FIGURE 1

2/7

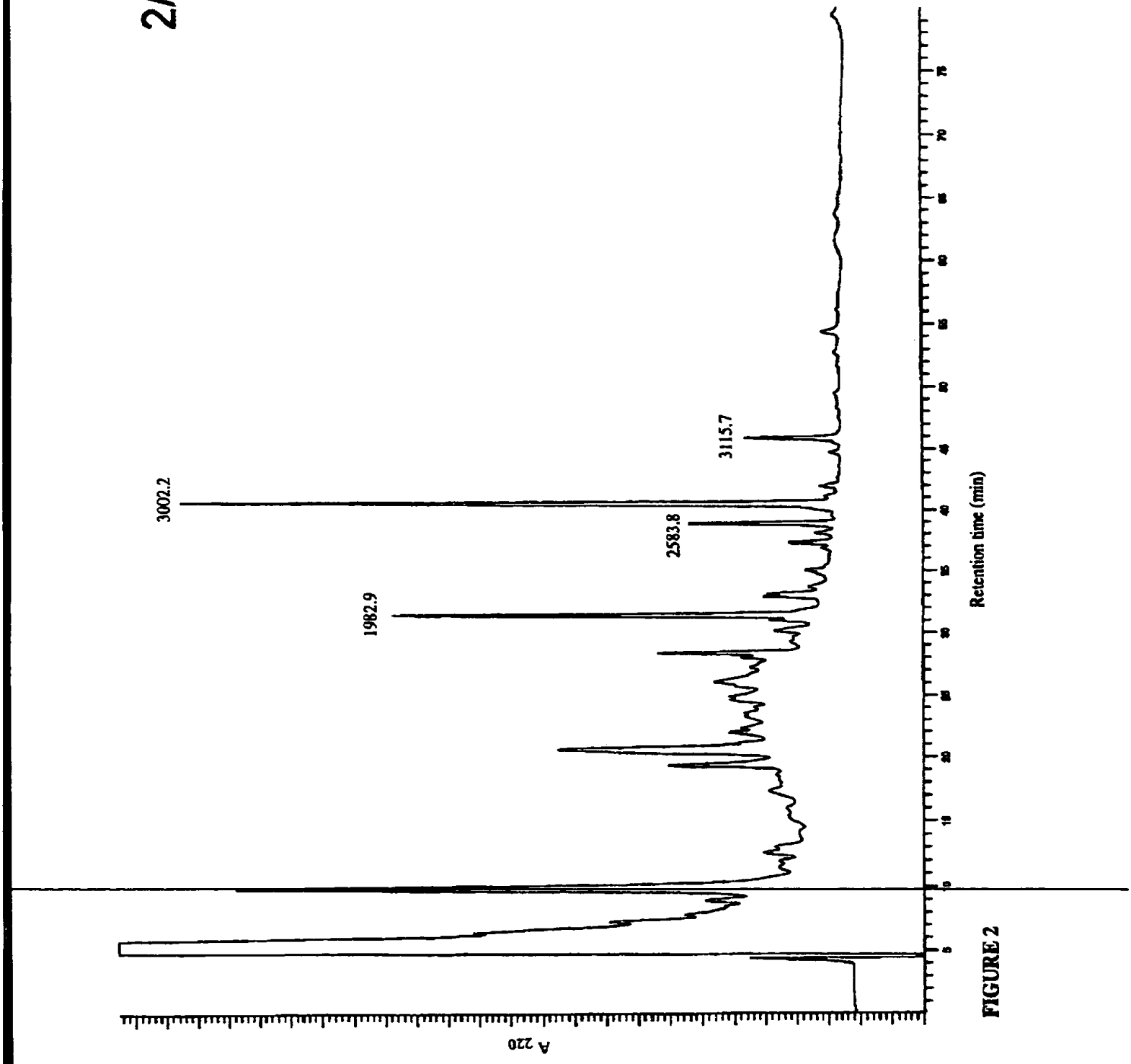
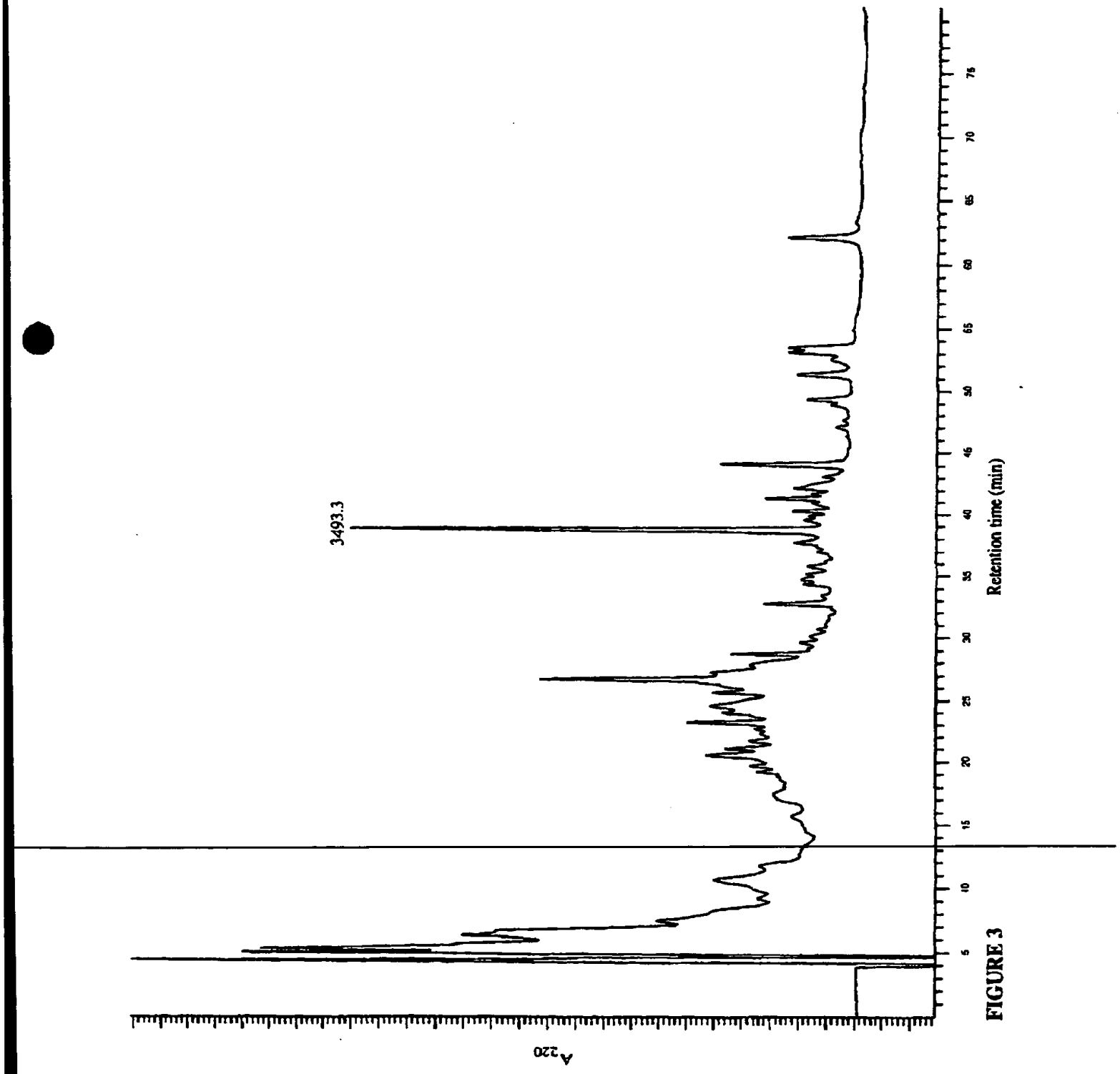


FIGURE 2



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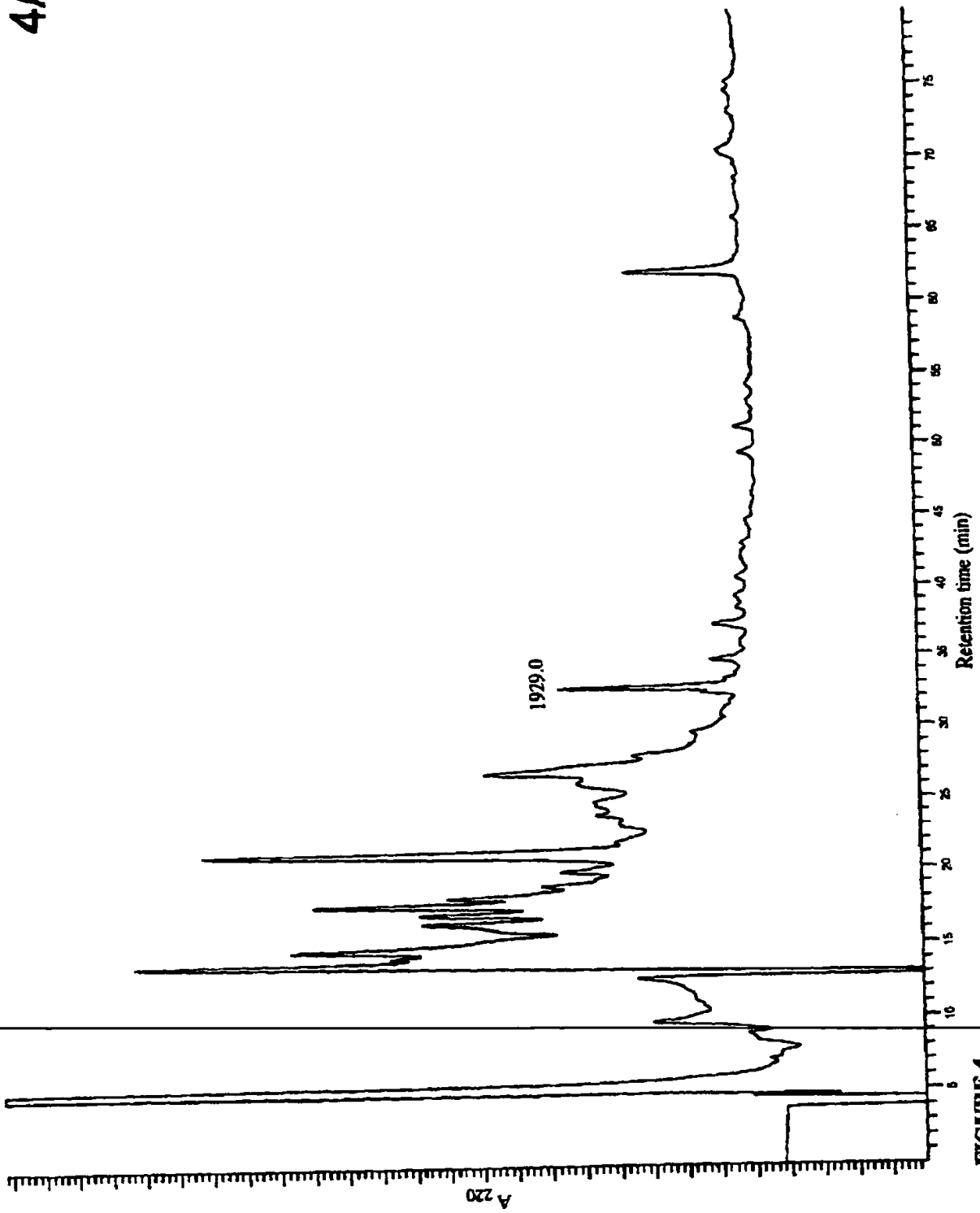

FIGURE 4

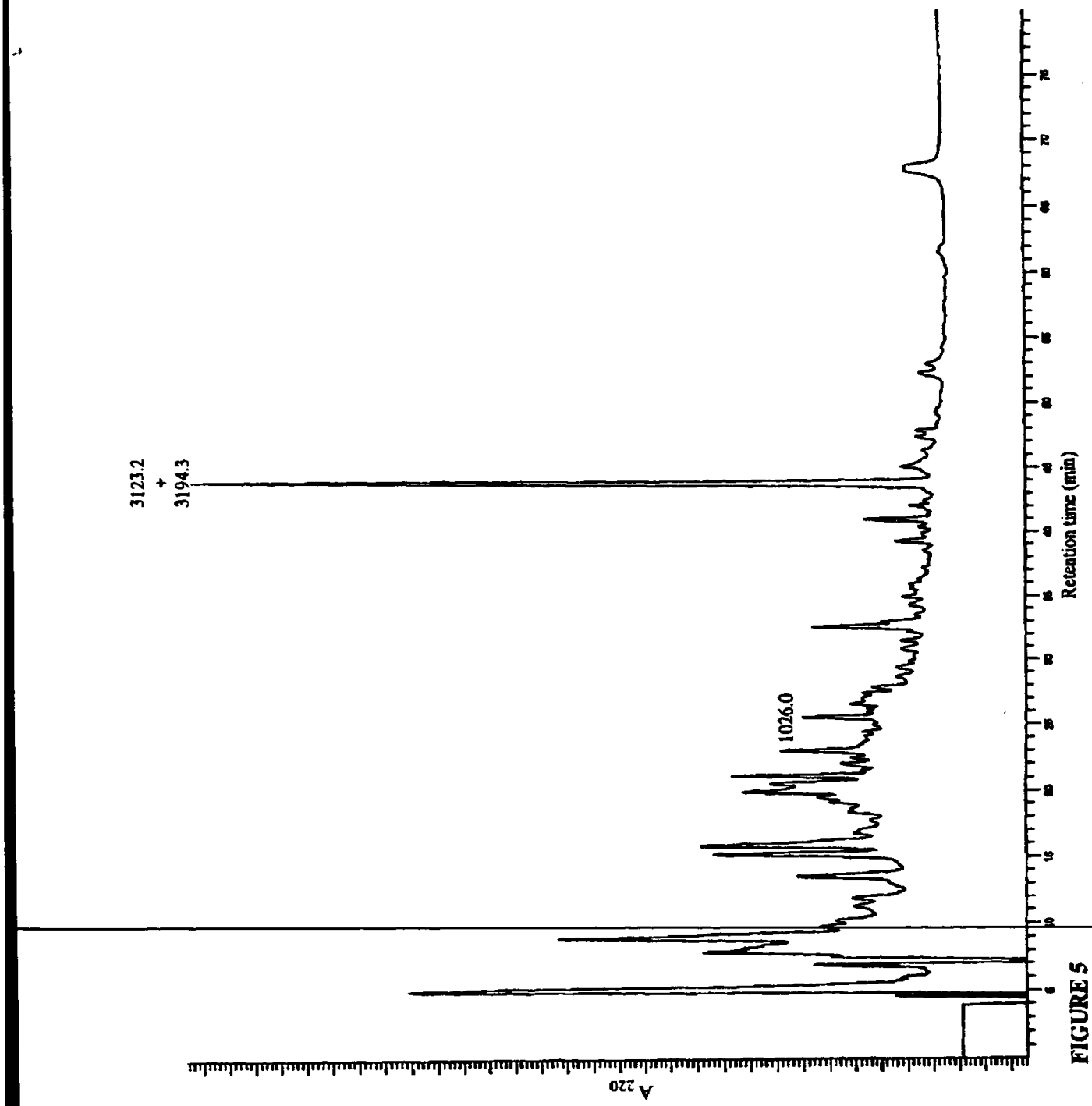

FIGURE 5

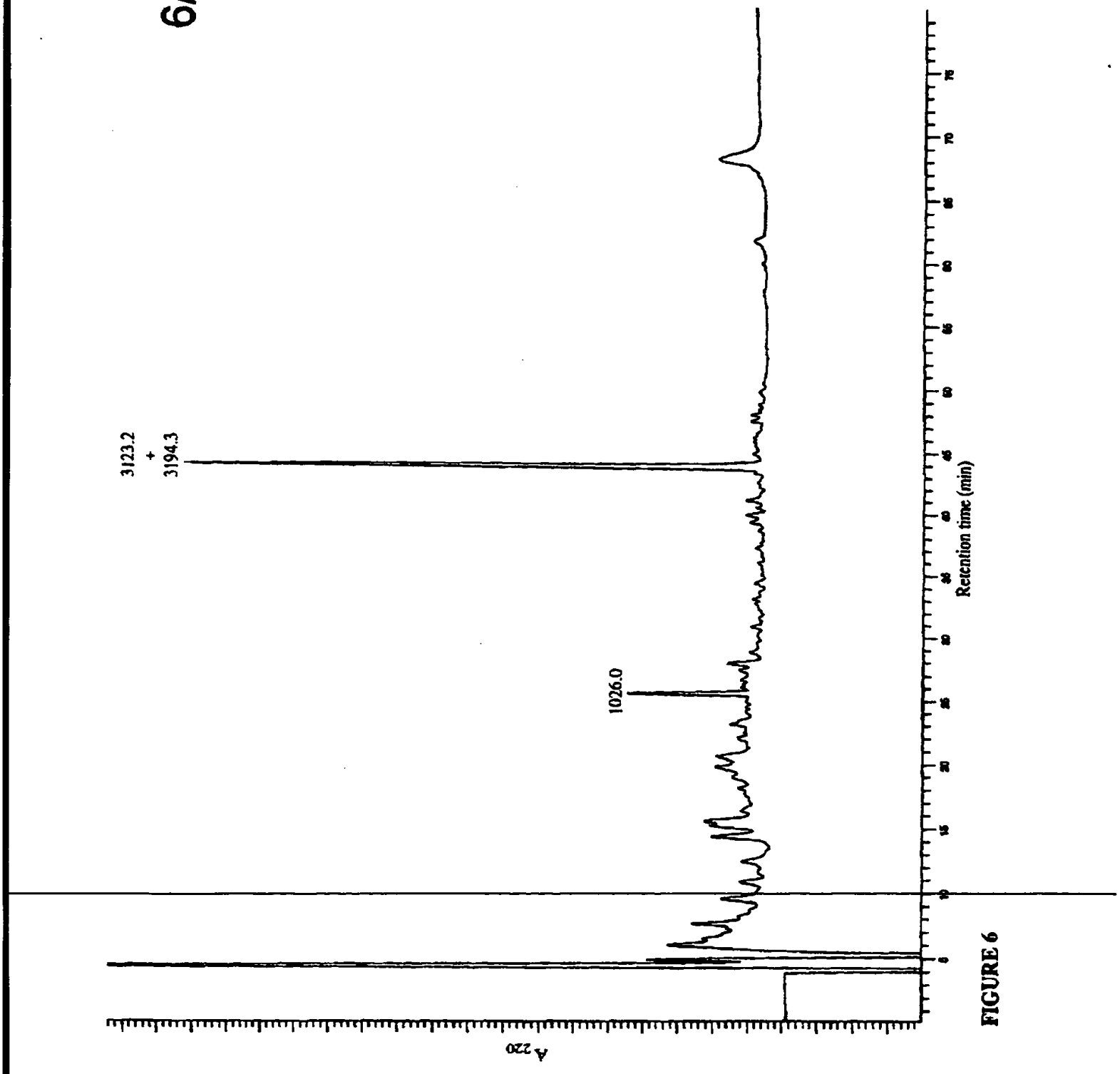

FIGURE 6

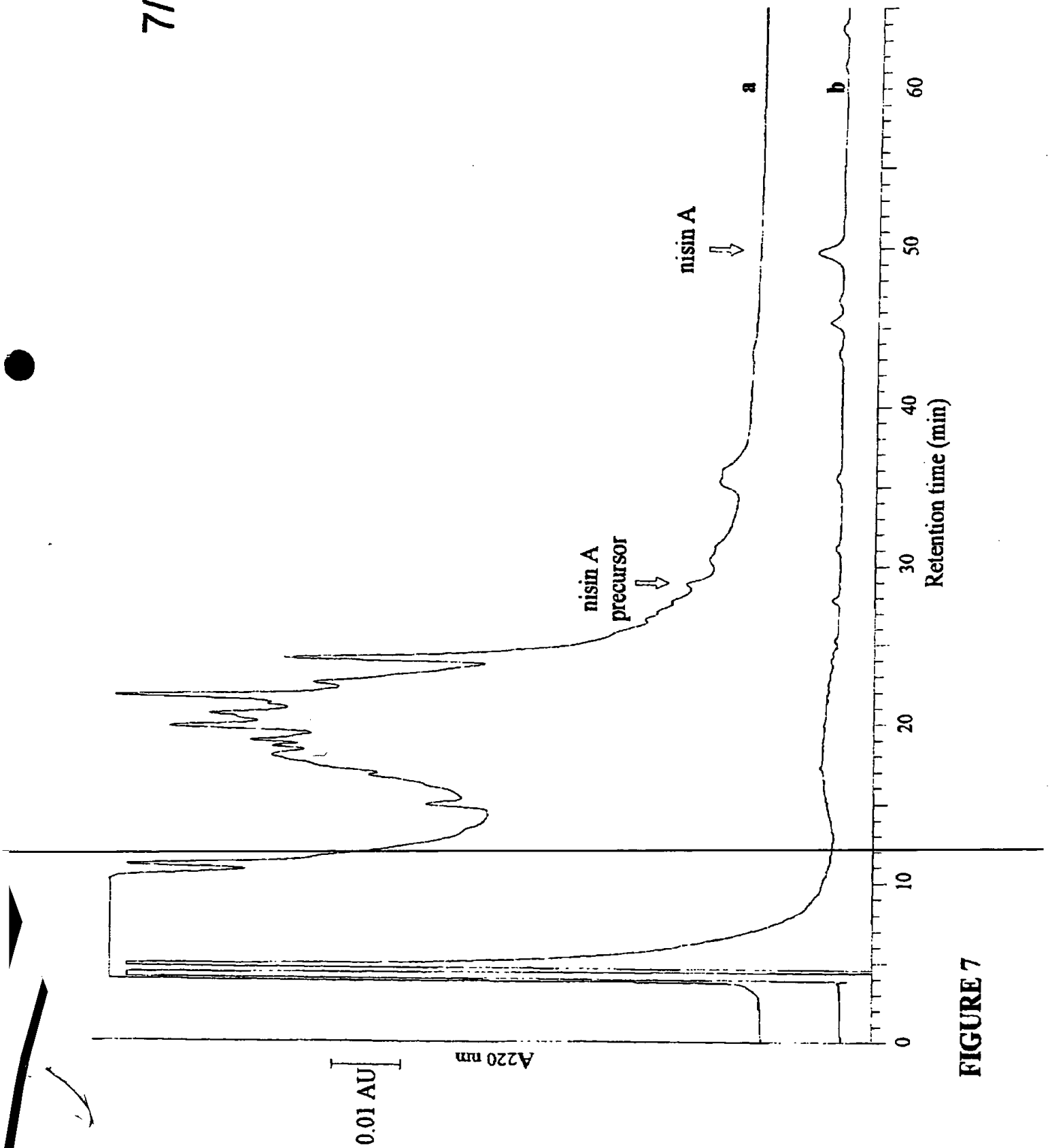
A220 nm
0.01 AU
nisin A
precursor
nisin A
a
b
0
10
20
30
40
50
60
Retention time (min)

FIGURE 7